The differences in metabolites in PBMC and in the plasma of patients with prediabetes or T2D compared to healthy controls

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ABSTRACT

The differences in metabolites in PBMC and in the plasma of patients with prediabetes or T2D compared to healthy controls

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To determine differences between peripheral blood mononuclear cells (PBMC) and the plasma matabolomes in patients with prediabetes or type 2 diabetes and healthy controls. 65 nononobese patients with prediabetes or newly-diagnosed type 2 diabetes and 65 nonobese sex-matched healthy controls were enrolled from an institutional health screen. After adjusting for age, BMI, blood pressure, and serum lipid profiles, the diabetic patients showed higher blood circulation and PBMC lipoprotein phospholipase A_2 (Lp-PLA_2) activities, C-reactive protein (hs-CRP), and TNF-α than controls. Compared with controls, diabetic subjects showed increases in 11 PBMC metabolites: six amino acids, L-pyroglutamic acid, two fatty

acid amides containing palmitic amide, oleamide, and two lysoPCs. In diabetic patients, PBMC Lp-PLA_2 significantly and positively associated with PBMC lysoPCs and inflammatory markers, including TNF-α, hs-CRP, and Lp-PLA_2 activities. When we compared the plasma metabolome of the patients and healthy controls, we observed significant increases in only two amino acids and decreases in only five lysoPCs. This study demonstrates significant differences in the PBMC metabolome in patients with prediabetes or diabetes compared with healthy controls. These differences were greater than those observed in the plasma metabolome. These data suggest PBMCs as a useful tool to better understand the inflammatory pathophysiology of diabetes.

Keywords : type 2 diabetes, prediabetes, PBMC metabolites, plasma metabolites, inflammatory markers, Lp-PLA_2 , TNF-α, lysoPCs, hs-CR

1. Introduction

A number of studies have reported on the metabolic patterns in prediabetic or diabetic patients (1-6). In a nested case-control study design, the Framingham Offspring study found that three branched chain amino acids (isoleucine, leucine, and valine) and two aromatic amino acids (tyrosine and phenylalanine) could predict the future development of diabetes in otherwise healthy individuals (1). Xu et al. recently reported that impaired fasting glucose (IFG) and type 2 diabetes (T2D) are characterized by abnormalities in amino acids, fatty acids, glycerophospholipids, and sphinglomyelin metabolism (2). In addition, Menni et al. found a novel association between the branched-chain keto acid metabolite, 3-methyl-2-oxovalerate, and IFG in both plasma and urine (3).

In early onset T2D, peripheral blood mononuclear cells (PBMC) are sensitive to a variety of stimuli; thus, individuals with T2D show a greater inflammatory reaction of PBMC than those without diabetes (7). PBMC consist of monocytes and lymphocytes. Blood monocytes play a key role in the onset and development of the inflammatory reaction by generating bioactive molecules, such as proinflammatory cytokines and lipoprotein phospholipase A_2 (Lp-PLA_2) (7, 8). Inflammatory processes have been increasingly recognized as a critical step in the pathogenesis of diabetes (9). Therefore, measuring the PBMC metabolome could provide a novel source of IFG/T2D biomarkers (10). The aim of this study was to assess the differences in metabolites in PBMC and in the plasma of patients with prediabetes or T2D compared to healthy controls.

2. Background

2.1. Diabetes

2.1.1. Type 2 diabetes

T2D is one of the most common chronic worldwide diseases; as a result prevalence of T2D is increasing at epidemic proportions. Population growth, dramatic increase in average life expectancy, and urbanization with associated changed life style are likely to lead to a 54% increase in worldwide numbers with diabetes by 2030 (11). This increase in T2D prevalence is also associated with increase in both morbidity and mortality (12). Mortality rate from diabetes has increased rapidly from 7.4% in 1988 to 25% in 2003 and has remained at approximately 20% since that time (13). Furthermore diabetic complications such as neuropathy, retinopathy, nephropathy and coronary diseases have a substantial and direct impact on medical costs (14).

T2D formerly called non insulin dependent diabetes mellitus, It is characterized by defects in both insulin secretion and insulin action (15). The risk of developing T2D increases with age, obesity, physical inactivity (16). Recent clinical trials have demonstrated that, changes in lifestyle and pharmacological intervention both reduces the incidence of diabetes with persons at high risk. Surprisingly, the lifestyle intervention shows more effective result than pharmacological treatment (17).

Below are 4 criteria for the diagnosis of diabetes according to the American Diabetes Association (ADA) (16).

1. glycated haemoglobin A_{1C} (HbA_{1C}) ≥ 6.5%
2. fasting plasma glucose (FPG) ≥ 126mg/dl

   (Fasting is defined as no caloric intake for at least 8h)
3. 2-h plasma glucose ≥ 200mg/dl during on oral glucose tolerance test (OGGT)
4. A patients with classic symptoms of hyperglycemia or hyperglycemic crisis with a random plasma glucose ≥ 200mg/dl

2.1.2. prediabetes

Subjects with prediabetes have high risk for the future development of diabetes. 5~10% of people per year with prediabets will progress to diabetes (18).

According to the ADA, people are at risk of developing diabetes if they have one of two distinct states: One is a IFG, defined as a FPG concentration 100 to 125mg/dl, without impaired glucose tolerance (IGT), and second is a IGT concentration 140 to 199mg/dl, measured during a 75g OGGT. And HbA_{1C} 5.7-6.4% as a category for high diabetes risk (16).

Prediabetes should be treated to prevent progression to diabetes. Most studies have focused on diabetes incidence in prediabetic individuals, and support opinion that change in lifestyle should be the first option for diabetes prevention (18).

The primary aim of lifestyle interventions is to prevent or delay development of T2D and its complication (19) by targeting obesity and physical inactivity, which are the two most important modifiable risk factor of diabetes development (20).

2.2. Metabolites

Metabolomics is defined as the quantitative measurement of the time-related multiparametric metabolomic response of living systems to pathophysiological stimuli or genetic modification. Based on the multivariate analysis of complex biological profiles, metabolomics has recently demonstrated enormous potential in many fields, such as disease diagnosis and toxicological mechanism and drug effects (21). Metabolites are small molecules that participate in metabolic reactions and have an important role in biological systems (22). Metabolomics studies can be performed using blood (serum, plasma), urine, cerebrospinal fluid, lymph fluid, bile, feces, saliva, cells, tissues and tissue or cell metabolic foot prints (22). Several studies have championed the potential use of PBMC, a group of cells, including lymphocytes and monocytes/macrophages, in characterizing gene expression patterns distinctive for certain disease (23).

Metabolic profiling of PBMC could serve as a less invasive and more direct alternative to tissue biopsies or serum in metabolomic research. The utility of metabolomic profiling of PBMCs and paves the way for future applications of metabolomics in identifying metabolic profiles of blood cells as a measure for dietary intake of physiological status (24).

2.3. Inflammtory markers & Type 2 diabetes

2.3.1. Lipoprotein-associated phospholipase A_2

Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) is a member of the phospholipase A_2 superfamily, a family of enzymes that hydrolyze phospholipids. Lp-PLA_2 is a proinflammatory enzyme secreted by macrophages that is primarily bound to low density lipoprotein (LDL) in the circulation. It hydrolyzes oxidized phospholipids to generate lysophosphatidylcholine and oxidixed fatty acid, which have proinflammatory properties, and its activity is increased in small dense LDL (25).

Levels of Lp-PLA_2 activity were significantly associated with incident coronary heart disease (CHD) among men and women with T2D, independent of traditional and inflammatory risk factors (26).

2.3.2. Tumor necrosis factor-α

Tumor necrosis factor-α (TNF-α), a paracrine/autocrine factor highly expressed in adipose tissues of obese animals and human subjects, is implicated in the induction of insulin resistance seen in obesity and T2D (27). Other studies have demonstrated synthesis of TNF-α in muscle and adipose tissue also have implicated TNF-α in the pathogenesis of insulin resistance (IR) (28). Concentrations of acute-phase response markers and mediators of inflammation – cytokines such as TNF-α and interleukin-6 (IL-6) are raised in people with T2D. An association between high plasma levels of TNF-α and several metabolic abnormalities characteristic for the insulin resistance syndrome suggests that TNF-α may be involved in the

pathogenesis of T2D(29).

2.3.3. Lysophophatidyl choline

Lysophophatidyl choline (Lyso-PC) plays a critical role in atherogenesis. It acts a chemoatractant for monocytes, impairs endothelial function, cause cell death by disrupting plasma membranes, and induces apoptosis in smooth muscle cells and macrophages (30,31). Others have reported increased lyso-PC content in LDL in patients with diabetes mellitus compared with non diabetic control (32). The lyso-PC content in LDL correlated with serum LP-PLA_2 level, and increased lyso-PC in LDL was associated with microangiopathy in diabetic patients (33).

2.3.4. C-reactive protein

C-reactive protein (CRP) is an acute-phase reactant that is part of the immune response to injury and infection (34). CRP is a marker of low-grade inflammation and may have indirect influence on insulin resistance and insulin secretion through altered innate immune response due to heightened systemic inflammation (35). Elevated CRP levels are a strong independent predictor of T2D (36). The magnitude of association between CRP and diabetes appears to be comparable or even stronger than the association of CRP with coronary heart disease observed in previous studies (37).

3. Materials and Methods

3.1 Subjects and Study Design

Study subjects were selected from participants in a clinical nutrition study conducted by the National Leading Research Laboratory of Clinical Nutrigenetics/Nutrigenomics at Yonsei University. Nonobese subjects ($18.5 \leq$ BMI < 30 kg/m^2), 30-70 years old, were recruited from the Health Service Center (HSC) during routine check-ups at the National Health Insurance Corporation Ilsan Hospital, Goyang, Korea (January 2013–June 2013). Based on the data screened from HSC, subjects who were in IFG ($100 \leq$ fasting glucose < 126 mg/dL) or newly diagnosed T2D (fasting glucose ≥ 126 mg/dL) were referred to the Department of Family Medicine or Internal Medicine. Subjects were excluded if they took: lipid-lowering medications, any medications or supplements known to affect lipid metabolism, or any probiotics products in the past month. Subjects were excluded if they were diagnosed with dyslipidemia, diabetes mellitus, hypertension, liver disease, renal disease, cardiovascular disease, cerebrovascular disease, pancreatitis, or cancer; history of medication or alcohol abuse; or were pregnant or breast feeding. A total of 65 nonobese subjects with prediabetes or newly diagnosed T2D (age 30-70 years) were enrolled, and 65 nonobese sex-matched healthy subjects were enrolled for the control group. The macronutrient composition of each subject's usual diet was that of a typical diet, which is consumed by a substantial number of Koreans. This diet derives about 63% of its energy from carbohydrates, 21% from fat, and 16% from protein. All subjects

provided written informed consent before participation in this study, which was approved by the Institutional Review Board of Yonsei University and Ilsan Hospital.

3.2 Anthropometric parameters, blood pressure, and blood collection

Weight and height were measured without clothes and shoes, and BMI was calculated by kilograms per square meter (kg/m^2). Waist and hip circumferences were measured using a plastic measuring tape and used to calculate the waist to hip ratio (WHR). After a 20-minute rest, blood pressure (BP) was measured two times in the left arm of seated subjects with an automatic BP monitor (FT-200S, Jawon Medical, Gyeongsan, Korea). After a 12-hour fasting period, venous blood specimens were collected in EDTA-treated whole blood and serum tubes. The blood samples were centrifuged to obtain plasma and serum. The collected samples were stored at -70℃.

3.3 Fasting glucose, insulin, homeostasis-model assessment of insulin resistance, and hemoglobin A_{1c}

Fasting glucose levels were analyzed by the hexokinase method using a Hitachi 7600 Autoanalyzer. Insulin levels were measured by an immunoradiometric assay kit from DIAsource ImmunoAssays S.A. (Louvain, Belgium). IR was calculated by the homeostasis-model assessment (HOMA) using the following equation: HOMA-IR = [Fasting insulin (μIU/mL) × Fasting glucose (mmol/L)] / 22.5. Hemoglobin A_{1c} (HbA_{1c}) was measured by immonoturbidimetric analysis.

3.4 Serum lipid profile and free fatty acids

Fasting triglyceride and total cholesterol were measured using a Hitachi 7600 Autoanalyzer (Hitachi Ltd., Tokyo, Japan). HDL-cholesterol left in the supernatant fraction was measured by an enzymatic method after other lipoprotein precipitation. LDL-cholesterol was indirectly calculated using the Friedwald formula; LDL cholesterol = total cholesterol – [HDL cholesterol + (triglyceride/5)] for subjects with a serum triglyceride level < 400 mg/dL. LDL-cholesterol was measured directly using a Hitachi 7600 Autoanalyzer for subjects with a serum triglyceride level ≥ 400 mg/dL. Free fatty acids (FFA) were measured by enzymatic assay with the acylCoA synthetase-acylCoA oxidase (ACS-ACOD) method using a Hitachi 7600 Autoanalyzer.

3.5 Measurement of serum high-sensitivity C-reactive protein, Lp-PLA_2 activity, plasma malondialdehyde, LDL particle size, and oxidized LDL

Serum high-sensitivity C-reactive protein (hs-CRP) was measured with an ADVIA 2400 Clinical Chemistry System (Siemens Ltd., Tarrytown, NY) using a commercially available, high-sensitivity CRP-Latex(II) X2 kit (Denka-Seiken Co., Ltd., Tokyo, Japan). Lp-PLA_2 activity was measured using a modified high-throughput radiometric activity assay (38). Plasma malondialdehyde (MDA) was measured from thiobarbituric acid-reactive substances (TBARS) using a TBARS Assay Kit (ZeptoMetrix Co., Buffalo, NY). LDL particles were isolated by sequential flotation ultracentrifugation. Particle size distribution (1.019–1.063 g/mL) was examined using a pore-gradient lipoprotein system (CBS Scientific

Company, San Diego, CA) using commercially available, non-denaturing gels containing a linear 2-16% acrylamide gradient (CBS Scientific Company). Latex-bead (30 nm) conjugated thyroglobulin (17 nm), ferritin (12.2 nm), and catalase (10.4 nm) standards were used to estimate the relative band migration rates. Gels were scanned using a GS-800 Calibrated Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA). Plasma oxidized (ox)-LDL was measured using an enzyme immunoassay (Mercodia AB, Uppsala, Sweden), and the resulting color reaction was determined at 450 nm on a Wallac Victor2 multilabel counter (Perkin-Elmer Life Sciences, Boston, MA).

3.6 Urinary 8-epi-prostaglandin $F_{2\alpha}$ and TNF-α measurements

Urine was collected in polyethylene bottles containing 1% butylated hydroxytoluene after a 12-h fast, and bottles were immediately covered with aluminum foil and stored at −20°C until further analysis. Levels of 8-epi-prostaglandin $F_{2\alpha}$ (8-epi-$PGF_{2\alpha}$) were measured using a Urinary Isoprostane ELISA kit (Oxford Biomedical Research Inc., Rochester Hills, MI). Urinary creatinine levels were determined by the Creatinine Jaffe Method. Serum TNF-α concentrations were measured using the Bio-Plex Reagent Kit and a Bio-Plex (Bio-Rad Laboratories, Inc.) according to manufacturer instructions.

3.7 Global (nontargeted) metabolic profiling of plasma

Sample preparation and analysis

Prior to analysis, 800 μL of 80% acetonitrile was added to 100 μL of plasma, mixed by vortexing, and centrifuged at 10,000 rpm for 5 min at 4℃. The supernatant was dried with N_2 (l), dissolved in 10% methanol, mixed by vortexing, and centrifuged at 10,000 rpm for 5 min at 4℃. The supernatant was transferred into a vial.

Ultra performance liquid chromatography

Plasma extract samples (4 μL) were injected into an Acquity UPLC-BEH-C18 column (2.1 × 50 mm, 1.7 μm; Waters, Milford, MA), which was coupled in-line with a UPLC-LTQ-Orbitrap XL (Thermo Fisher Scientific, USA). The injected samples were equilibrated with water containing 0.1% formic acid. Samples were eluted with an acetonitrile gradient containing 0.1% formic acid at a flow rate of 0.35 mL/min for 20 min. Metabolites were separated by UPLC (Thermo Fisher Scientific, USA), analyzed, and assigned by LTQ-Orbitrap-XL (Thermo Fisher Scientific, USA). The mass spectrometer was operated in the ESI-positive mode. The spray voltage was 5 kV. The flow rate nitrogen sheath gas and the auxiliary gas were 50 and 5 (arbitrary units). The capillary voltage (V), tube-lens voltage (V), and capillary temperature (℃) were kept constant at 35 V, 80 V, and 370℃. The Orbitrap data were collected in the range of *m*/*z* 50–1,000. The MS/MS spectra of metabolites were obtained by a collision-energy ramp from 55–65 eV, and conducted with Xcalibur 2.1 and MS Frontier software (Thermo Fisher Scientific, USA).

Data processing and identification of metabolites

All MS data including retention times, *m*/*z*, and ion intensities were extracted by SIEVE software (Thermo Fisher Scientific, USA) incorporated into the instrument, and the resulting MS data were assembled into a matrix. SIEVE parameters were set as follows: *m*/*z* range 50–1,000; *m*/*z* width 0.02; retention time width 2.5; and *m*/*z* tolerance 0.005. Metabolites were searched using the following databases: ChemSpider (www.chemspider.com), Human Metabolome (www.hmdb.ca), Lipid MAPS (www.lipidmaps.org), KEGG (www.genome.jp/kegg), and MassBank (www.massbank.jp). Selected metabolites were confirmed by standard samples based on both retention times and mass spectra.

3.8 Statistical analyses

Statistical analyses were performed using SPSS v. 21.0 software (IBM/SPSS Corp. Chicago, IL). Skewed variables were logarithmically transformed for statistical analyses. For descriptive purposes, mean values were presented using untransformed values. Results were expressed as means ± standard error (SE). A two-tailed P-value of < 0.05 was considered statistically significant. Differences in biochemical characteristics between two groups were tested using Student's independent *t*-test. General linear model (GLM) tests were applied to compare parameter changes between the two groups by adjusting for baseline values. Pearson's and partial correlation coefficients were used to examine the relationships between variables over time. False discovery rate (FDR)-corrected *q*-values were computed using the R package 'fdrtool'. Heat maps were created to

visualize and evaluate correlations among metabolites and biochemical characteristics in the study populations.

Multivariate statistical analysis was performed using SIMCA-P+ software version 12.0 (Umetrics, Umeå, Sweden). Partial least-squares discriminant analysis (PLS-DA) was used as the classification method for modeling the discrimination between healthy controls and subjects with IFG or T2D by visualizing the score plot or *S*-plot using the first and second PLS components. To validate the model, a seven-fold validation was applied to the PLS-DA model, and the reliabilities of the model were further rigorously validated by a permutation test (n = 200). The goodness of the fit was quantified by R^2Y, whereas the predictive ability was quantified by Q^2Y. Generally, R^2Y describes how well the data in the training set are mathematically reproduced, and this varies between 0 and 1 (a value of 1 indicates a model with a perfect fit). Models with $Q^2Y \geq 0.5$ are considered to have good predictive capabilities.

4. Results

4.1 Clinical characteristics and nutrient intake

Clinical and biochemical characteristics of healthy controls with normal fasting serum glucose level and subjects with IFG or newly diagnosed T2D are shown in Table 1. There were no significant differences in clinical characteristics, such as male/female distribution (25/40 vs. 25/40), education level, smoking, drinking, and total calorie and macronutrient intake (data not shown) between control and patient groups. The patient group was significantly older and heavier and had higher diastolic BP, serum triglyceride, and FFA compared to controls. After adjusting for age, BMI, BP, and serum lipid profiles, when compared with control subjects those with IFG or T2D showed higher glucose, HbA_{1C}, insulin, HOMA-IR, hs-CRP, circulating plasma and PBMC Lp-PLA_2 activities, ox-LDL, 8-epi-$PGF_{2\alpha}$, MDA, and TNF-α. The patient group also had a smaller LDL particle size compared with controls (Table 1).

Table 1. Clinical and biochemical characteristics in each group

	NFG (n=65)	IFG + T2D (n=65)	*P*	P_1
Age (year)	48.2±1.12	54.0±1.00	<0.001	-
Body mass index (kg/m^2)	22.8±0.35	24.0±0.34	0.014	-
Waist hip ratio	0.87±0.01	0.90±0.01	0.008	-
Systolic BP (mmHg)	118.3±1.54	125.8±1.61	0.001	-
Diastolic BP (mmHg)	77.4±1.29	80.2±1.15	0.111	-
Triglyceride (mg/dL) [♯]	100.7±5.38	126.8±7.73	0.013	-
Total-cholesterol (mg/dL) [♯]	198.5±3.35	195.6±4.61	0.434	-
HDL-cholesterol (mg/dL) [♯]	54.4±1.84	48.3±1.34	0.023	-
LDL-cholesterol (mg/dL) [♯]	122.8±3.17	121.9±4.06	0.650	-
Free fatty acid (μEq/L) [♯]	389.2±24.5	488.5±23.5	0.001	-
Glucose (mg/dL) [♯]	88.7±1.06	119.9±3.59	<0.001	<0.001
HbA_{1c} (%) [♯]	5.76±0.01	6.47±0.09	<0.001	<0.001
Insulin (μIU/dL) [♯]	7.01±0.77	7.58±0.42	0.032	0.009
HOMA-IR [♯]	1.54±0.17	2.22±0.13	<0.001	<0.001
hs-CRP (mg/dL) [♯]	0.45±0.14	1.73±0.36	<0.001	<0.001
LDL particle size (nm) [♯]	23.5±0.08	23.0±0.11	0.001	0.012
Lp-PLA_2 activity (nmol/mL/min) [♯]	26.8±0.75	31.7±0.74	<0.001	<0.001
PBMC Lp-PLA_2 activity (nmol/mL/min) [♯]	1.47±0.05	2.07±0.10	<0.001	<0.001
Oxidized LDL (U/L) [♯]	40.8±1.48	53.9±1.91	<0.001	<0.001
8-epi-$PGF_{2\alpha}$ (pg/mg creatinine) [♯]	1156.1±50.6	1833.6±81.2	<0.001	<0.001
Malondialdehyde (nmol/mL) [♯]	7.88±0.28	8.97±0.29	0.002	0.010
Serum TNF-α (pg/mL) [♯]	7.31±1.23	12.3±1.83	<0.001	0.001

Mean ± SE. [♯] Tested by logarithmic transformation, *P*-values derived from independent *t*-test. P_1 adjusting for age, BMI, WHR, smoking, drinking, BP, triglyceride, total cholesterol, HDL, LDL, and FFA

4.2 PBMC and plasma metabolic profiling using UPLC-LTQ-Orbitrap mass spectrometry

Nontargeted metabolic pattern analysis

The mass spectrometry (MS) data of PBMC metabolites were analyzed with a PLS-DA score plot (Fig. 1A). The two-component PLS-DA score plot of the PBMC metabolites showed distinct clustering and clear separation for each of the healthy controls away from those subjects with IFG or T2D. Both groups could be clearly differentiated from each other by the primary component $t(1)$ or the secondary component $t(2)$ based on the model with R^2X(cum) and R^2Y(cum) values of 0.256 and 0.682, respectively, indicating a good fit of the data. The Q^2Y(cum) value of 0.608 provides an estimate of the predictive ability of this model. The PLS-DA model was validated using a permutation test, which indicated an R^2Y intercept value of 0.258 and a Q^2Y intercept value of –0.185.

The MS data of plasma metabolites were analyzed with a PLS-DA score plot (Fig. 1B). The two-component PLS-DA score plot of the plasma metabolites showed distinct clustering and clear separation for each of the healthy controls away from that observed for subjects with IFG or type 2 diabetes. Both groups could be clearly differentiated from each other by the primary component $t(1)$ or the secondary component $t(2)$ based on the model with R^2X(cum) and R^2Y(cum) values of 0.12 and 0.708, respectively, indicating a good fit of the data. The Q^2Y(cum) value of 0.414 provides an estimate of the predictive ability of the model. The PLS-DA model was validated using a permutation test, which indicated an R^2Y intercept value of 0.486 and a Q^2Y intercept value of –0.0496. To identify the

metabolites that contributed to the differentiation between healthy controls and IFG or diabetic subjects, *S*-plots of *p*(1) and *p*(corr)(1) were generated using centroid scaling (Fig. 1 C and D). The *S*-plots revealed that metabolites with higher or lower *p*(corr) values were more relevant for discriminating between the two groups.

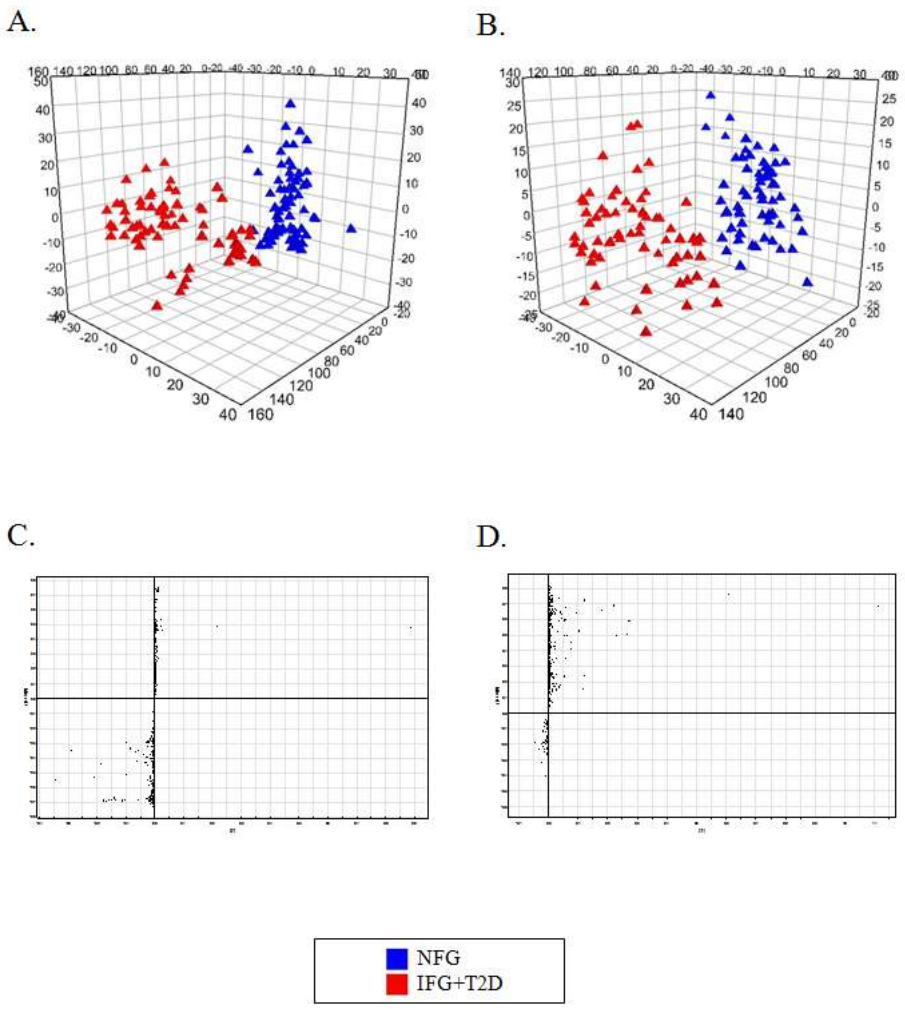

Figure 1.

Identification of PBMC and plasma metabolites with significantly altered levels in each subgroup. A: Score plots of PBMC metabolites from PLS-DA models classifying in NFG (*n*=65) and IFG or T2D group (*n*=65). B: Score plots of plasma metabolites from PLS-DA models classifying in NFG (*n*=65) and IFG or T2D group (*n*=65). C&D: S-plots for covariance [*p*], and the reliability correlation

[*p*(corr)] from PLS-DA models.

Identification of PBMC metabolites

Of 1,948 PBMC metabolites, those that correlated with a separation between the groups were identified by the variable important in the projection (VIP) parameter. VIP values > 1.0 were considered highly relevant for group differences. Thirty-six metabolites were selected based on VIP values > 1.0; 11 of these were previously identified, and 25 were unknown. Eleven PBMC metabolites (VIP > 1.0) are shown in Table 2. The normalized peak intensities of 6 amino acids (valine, leucine, methionine, phenylalanine, tyrosine, tryptophan) were significantly higher in PBMCs of IFG or diabetic subjects compared to those of control subjects (all *q*-values < 0.001). L-pyroglutamic acid, two fatty acid amides containing palmitic amide and oleamide, and two lysoPC containing C16:0, C18:0 were significantly higher in PBMCs of IFG or diabetic subjects than in those of control subjects (Table 2).

Table 2. Identification of PBMC metabolites in each group

Identity	Formula $[M+H]^+$	Exact Mass (M+H)	Normalized peak intensities		*q*-value	VIP
			NFG (n=65)	IFG + T2D (n=65)		
L-Valine	$C_5H_{11}NO_2$	118.0868	888316±37074	1057811±30070	$4.2X10^{-4}$	1.3129
L-Pyroglutamic acid	$C_5H_7NO_3$	130.0504	1768814±108739	2440611±84749	$4.2X10^{-6}$	5.0910
L-Leucine	$C_6H_{13}NO_2$	132.1024	5845735±146747	7718791±205605	$<1.0X10^{-9}$	14.4867
L-Methionine	$C_5H_{11}NO_2S$	150.0589	572911±24036	786394±26709	$5.8X10^{-8}$	1.6523
L-Phenylalanine	$C_9H_{11}NO_2$	166.0868	2118750±74031	2703044±64325	$4.0X10^{-8}$	4.5446
L-Tyrosine	$C_9H_{11}NO_3$	182.0817	1197246±34486	1508002±36979	$2.1X10^{-8}$	2.4183
L-Tryptophan	$C_{11}H_{12}N_2O_2$	205.0977	674782±18517	813131±20492	$2.5X10^{-6}$	1.0979
Palmitic amide	$C_{16}H_{33}NO$	256.2640	189455±36005	416239±64030	$1.6X10^{-3}$	1.7109
Oleamide	$C_{18}H_{35}NO$	282.2797	1813863±371626	3295742±501718	$9.3X10^{-3}$	11.1721
LysoPC (16:0)	$C_{24}H_{50}NO_7P$	496.3403	308429±43686	487640±44173	$2.7X10^{-3}$	1.3651
LysoPC (18:0)	$C_{26}H_{54}NO_7P$	524.3716	247036±33381	399886±32969	$9.8X10^{-4}$	1.1729

Mean ± SE

Identification of plasma metabolites

Among 4,164 metabolites in the plasma, we identified those that played important roles in the separation between the groups by selecting according to the Variable Important in the Projection (VIP) parameter. VIP values > 1.0 were considered to be highly relevant for the generating the difference between the sample groups. Eighty-one plasma metabolites were selected based on VIP values > 1.0; 12 of these metabolites and PCs were previously identified, and 69 metabolites were unknown. Twelve plasma metabolites and PCs are shown in Table 3. The normalized peak intensities of two amino acids (proline, valine) were significantly higher in the plasma of IFG or diabetic subjects compared to the peak intensities observed in control subjects (both *q*-values <0.001). Five lysoPC containing C16:0, C17:0, C18:2, C18:1, and C18:0 were significantly lower in the plasma of IFG or diabetic subjects compared with control subjects (Table 3).

Table 3. Identification of plasma metabolites in each group

Identity	Formula $[M+H]^+$	Exact Mass (M+H)	Normalized peak intensities		*q*-value	VIP
			NFG (n=65)	IFG + T2D (n=65)		
L-Proline	$C_5H_9NO_2$	116.0712	276492±15880	414602±18452	8.6×10^{-7}	1.1046
L-Valine	$C_5H_{11}NO_2$	118.0868	1167581±37876	1302131±23979	8.8×10^{-3}	1.1138
L-Leucine	$C_6H_{13}NO_2$	132.1024	2692278±113534	3031235±65932	0.232	2.7660
L-Phenylalanine	$C_9H_{11}NO_2$	166.0868	1799375±58017	1939404±36325	0.066	1.1481
Oleamide	$C_{18}H_{35}NO$	282.2797	7273413±588229	7897810±644605	0.292	13.3814
LysoPC (16:0)	$C_{24}H_{50}NO_7P$	496.3403	18894117±658364	16807599±459092	0.022	16.9894
LysoPC (17:0)	$C_{25}H_{52}NO_7P$	510.3559	943675±63110	744399±35337	0.016	1.4135
LysoPC (18:2)	$C_{26}H_{50}NO_7P$	520.3403	6873739±259398	6005440±176424	0.016	6.0622
LysoPC (18:1)	$C_{26}H_{52}NO_7P$	522.3559	6027713±224255	5336388±160530	0.027	4.8448
LysoPC (18:0)	$C_{26}H_{54}NO_7P$	524.3716	6830842±261237	6019618±155138	0.019	5.7595
LysoPC (20:4)	$C_{28}H_{50}NO_7P$	544.3403	2048369±86854	2144722±91164	0.280	2.5718
Lactosylceramide (d18:1/12:0)	$C_{42}H_{79}NO_{13}$	806.5629	5824805±467128	6640208±481304	0.197	8.2140
[1]PCs			37066131±1525042	35406576±1441222	0.274	

Mean ± SE. [1]PCs were not successfully separated by C18-UPLC used in this study, but detected by Orbitrap MS. Thus, the amounts of all detected PCs were combined together.

4.4 Correlations among fasting glucose, plasma and PBMC Lp-PLA_2 activities, biochemical parameters, and major PBMC and plasma metabolites

The correlation matrix among fasting glucose, plasma and PBMC Lp-PLA_2 activities, biochemical parameters, and major PBMC and plasma metabolites was computed for all subjects (n = 130) as well as for the control and patient groups (Fig. 2). In all subjects, fasting glucose, plasma and PBMC Lp-PLA_2 activities, biochemical parameters, and major PBMC and plasma metabolites were highly correlated. For example, fasting glucose correlated positively with HOMA-IR, ox-LDL, plasma and PBMC Lp-PLA_2, hs-CRP, TNF-α, 8-epi-$PGF_{2\alpha}$, PBMC valine, L-pyroglutamic acid, leucine, methionine, phenylalanine, and tyrosine; plasma proline, valine, leucine, and phenylalanine. Fasting glucose negatively correlated with LDL particle size and plasma lysoPCs containing C17:0 and C18:2.

In the control group, fasting glucose positively correlated with PBMC valine and tryptophan. Plasma Lp-PLA_2 activity positively correlated with PBMC Lp-PLA_2 (r = 0.273, P = 0.032), phenylalanine, tyrosine, and lysoPC 18:0 (r = 0.251, P = 0.048). PBMC Lp-PLA_2 activity correlated positively with HOMA-IR, hs-CRP, PBMC leucine, methionine, phenylalanine, tyrosine, palmitic amide, oleamide, lysoPC (16:0) (r = 0.273, P = 0.030), lysoPC (18:0) (r = 0.266, P = 0.035); plasma valine, leucine, phenylalanine, oleamide, lysoPCs containing C16:0, C17:0, C18:2, C18:1, C18:0 and C20:4. Fasting glucose negatively correlated with LDL particle size.

In the patient group, plasma Lp-PLA_2 activity strongly and positively correlated with ox-LDL (r = 0.464, P < 0.001), 8-epi-$PGF_{2\alpha}$ (r = 0.500, P < 0.001), TNF-α,

and PBMC Lp-PLA_2 (r = 0.518, $P < 0.001$). PBMC Lp-PLA_2 activity positively correlated with hs-CRP, TNF-α, ox-LDL (r = 0.445, $P < 0.001$), PBMC phenylalanine, palmitic amide, oleamide, lysoPC 16:0 (r = 0.421, $P < 0.001$), and lysoPC 18:0 (r = 0.415, $P < 0.001$) (Fig. 2).

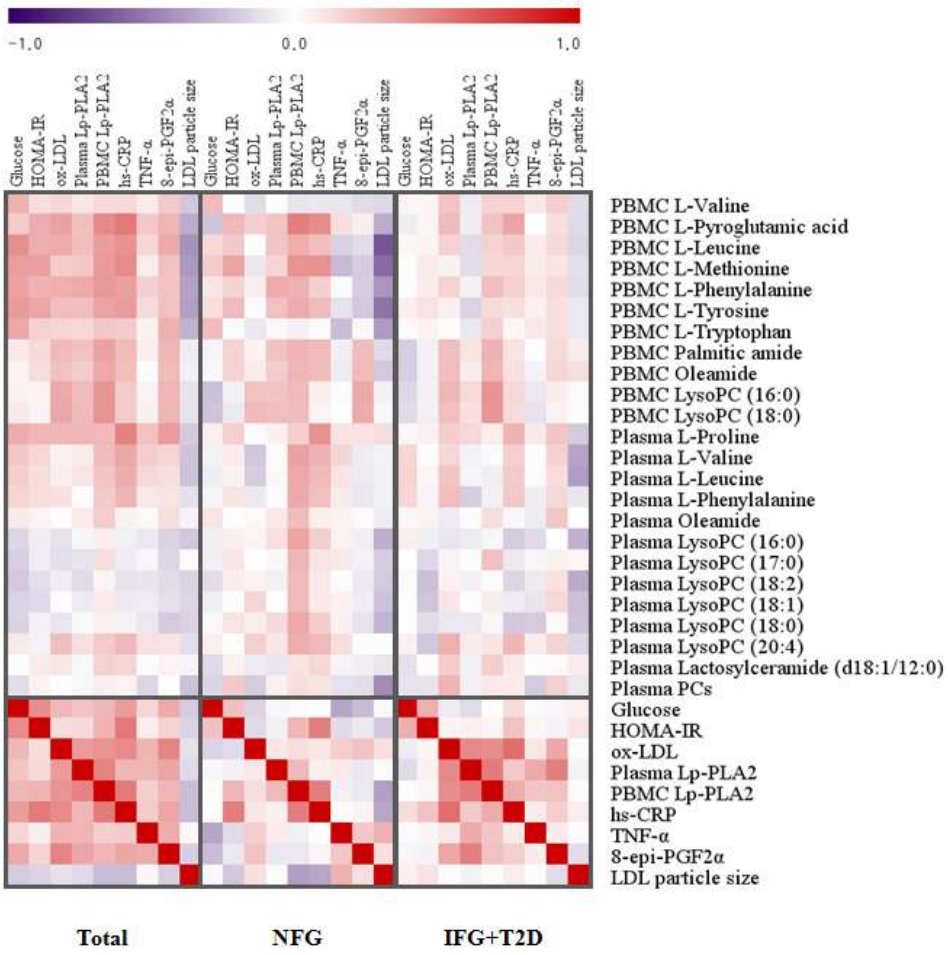

Figure 2.

Correlation matrix among metabolites and biochemical characteristics in each group. All biochemical characteristics were tested by logarithmic transformation. Correlations were obtained by deriving a Spearman correlation coefficient. PBMC metabolites (11), plasma metabolites (13), and biochemical characteristics (9) are listed on the right side of the heat map with the nine biochemical characteristics listed across the top. *Red* represents a positive correlation, and *purple* represents a negative correlation.

5. Discussion

The major finding of this study is that, compared with plasma metabolites, the PBMC metabolites show unique metabolomic features and have a greater difference between patients with prediabetes or T2D compared to healthy controls. Accounting for prediabetes- or diabetes-related alterations of PBMC and plasma major metabolites, we identified 11 PBMC metabolites to be significantly different between control and patient groups. These included six amino acids (valine, leucine, methionine, phenylalanine, tyrosine, tryptophan), L-pyroglutamic acid, palmitic amide, oleamide, and two lysoPCs containing C16:0, C18:0. Comparatively, there were significant differences in seven plasma metabolites, including two amino acids (proline and valine) and five lysoPCs containing C16:0, C17:0, C18:2, C18:1, C18:0. In patients with prediabetes or T2D, the positive associations among PBMC Lp-PLA2, PBMC lysoPCs, and circulating inflammatory markers (TNF-α, hs-CRP, Lp-PLA_2 activity) agrees with previous data indicating that PBMCs have a greater inflammatory reaction in individuals with T2D compared with those without T2D (7). Therefore, these results support the recent proposal that due to their active metabolism, PBMC may be useful to better understand the pathophysiology of chronic diseases (10, 39). Furthermore, these results indicate that subset of PBMC metabolites are unique from plasma metabolites, suggesting a possible enhanced effectiveness of using this approach compared to plasma metabolomics alone.

In a nested case-control study design, five plasma branched-chain and aromatic amino acids (isoleucine, leucine, valine, tyrosine and phenylalanine) had

significant associations with the development of diabetes (1). Our cross-sectional investigation also showed PBMC branched-chain and aromatic amino acids and methionine to be significantly elevated in subjects with IFG or T2D. Unlike PBMC metabolites, only two plasma amino acids, proline and valine, showed significant differences when comparing the control and patient groups. This discrepant finding may indicate divergent mechanisms used by different tissues to control metabolites. Pyroglutamic acid is important in the intracellular transport of free amino acids, and Xu et al. found serum pyroglutamic acid to be lower in patients with diabetes (2). In contrast, in the current study PBMC pyroglutamic acid was higher in subjects with IFG or T2D. Furthermore, we observed discrepancies between lysoPCs in plasma compared with that of PBMC. We detected lower lysoPCs in the plasma and higher lysoPCs in PBMC in subjects with IFG or T2D compared controls.

Compared with healthy patients, those patients with IFG or T2D showed an increase in plasma ox-LDL as well as elevations in PBMC Lp-PLA_2 activities and PBMC lysoPCs. The observed strong association between PBMC Lp-PLA_2 and ox-LDL supports previously published data demonstrating a direct effect of ox-LDL on the expression of Lp-PLA_2 in THP-1 monocytes (40). Indeed, plasma ox-LDL has been suggested to have a causal role in upregulating Lp-PLA_2 mRNA expression in PBMC of smokers (41). Lp-PLA_2 hydrolyzes oxidized phospholipids in LDL particles at the *sn*-2 position and produces bioactive oxidized free fatty acids and lysoPCs. In the present study, strong positive associations among plasma ox-LDL, PBMC Lp-PLA_2 activities, PBMC lysoPC (16:0), and PBMC lysoPC

(18:0) may indicate that plasma ox-LDL and PBMC Lp-PLA_2 activities are major determinants of PBMC lysoPC levels. Furthermore, a negative relationship of PBMC Lp-PLA_2 activities, PBMC lysoPC (16:0), and PBMC lysoPC (18:0) with LDL particle size may reflect the apparent binding preference of Lp-PLA_2 for small dense LDL (42). Similar to a recent report (43), this study also showed positive associations among fasting glucose, PBMC Lp-PLA_2, and plasma Lp-PLA_2 activities. In a porcine model of diabetes, Shi et al. found the up-regulation of Lp-PLA_2 mRNA expression in PBMC (44).

In this study, IFG or T2D-related decreases in most plasma lysoPCs were observed regardless of increases in plasma Lp-PLA_2 activities. This result may partly be explained by the previous finding of reduced serum activity of lecithin-cholesterol acyltransferase (LCAT) in T2D, which transfers a fatty acid from PC to cholesterol and also regulates revers cholesterol transport and HDL remodeling (45). In the plasma, significant amounts of lysoPCs and most saturated lysoPCs can be formed by the action of LCAT (46). Reducing LCAT activity in T2D patients was recently suggested to limit the adverse effects of enhanced circulating ox-LDL on HDL-cholesterol metabolism (45). This study also showed an increase in plasma ox-LDL along with decreases in both HDL-cholesterol and plasma lysoPCs containing C16:0, C17:0, C18:2, C18:1, and C18:0. Therefore, IFG or T2D-related decreases in most plasma lysoPCs may in part be due to decreased biosynthesis resulting from depressed serum LCAT activity rather than increased clearance from the circulation by tissues. Whereas LCAT activity was not measured in this study, this LCAT-dependent mechanism is a plausible hypothesis.

IFG or T2D-related increases in PBMC fatty acid primary amides, oleamide and palmitic amide, were observed. Oleamide was the first fatty acid primary amide to be identified as a signaling molecule, and it has been shown to participate in several signaling pathways (47). This study identified PBMC oleamide (VIP value of 11.1721) and PBMC leucine (VIP value of 14.4867) as the most important PBMC metabolites for evaluating the difference between healthy patients and subjects with IFG or T2D. Recently, Ha et al. (4) found that of the plasma metabolites, plasma oleamide was useful for distinguishing between nondiabetic and diabetic men (VIP value of 6.952). Our study is in agreement with these findings. We found plasma oleamide (VIP value of 13.3814) and plasma lysoPC 16:0 (VIP value of 16.9894) to be the most important plasma metabolites for indicating IFG or T2D.

A large number of metabolite markers were detected by UPLC-LTQ-Orbitrap MS in this study, but most of these metabolites remain unidentified. Large databases of endogenous biomolecules have not yet been constructed for use with LC-MS-based techniques for metabolomics research (48). Despite these limitations, our approach using UPLC-LTQ-Orbitrap MS-based metabolomics and multivariate data analysis showed increases in 11 PBMC metabolites in subjects with IFG or T2D, including six amino acids (valine, leucine, methionine, phenylalanine, tyrosine, tryptophan; all q-values <0.001), L-pyroglutamic acid, palmitic amide, oleamide, and two lysoPCs containing C16:0, C18:0. PBMC Lp-PLA_2 in prediabetic or diabetic individuals. These increases significantly and positively correlated with PBMC lysoPCs and circulating inflammatory markers, including

TNF-α, hs-CRP, and Lp-PLA_2 activities. However, when we compared the plasma of the prediabetic and diabetic patients with controls, we observed significant increases in the plasma of only two amino acids, proline and valine; we also observed decreases in five lysoPCs containing C16:0, C17:0, C18:2, C18:1, and C18:0. In conclusion, this study demonstrates greater differences between patients with prediabetes or T2D and healthy controls in the PBMC metabolome than those observed in the plasma metabolome. These data indicate that PBMCs may be a useful tool to better understand the inflammatory pathophysiology of IFG or diabetes.

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국문요약

당뇨 전단계 및 제 2형 당뇨 환자와 정상인의

말초혈액단핵구 및 혈장 대사체의 차이

당뇨 전단계 및 제 2형 당뇨 환자와 정상인의 말초혈액단핵구(PBMC)와 혈장 대사체의 차이점을 밝히기 위한 연구이다. 당뇨 전단계이거나 제 2형 당뇨로 새롭게 진단받은 65명의 환자와 성별이 동일한 65명의 비당뇨인이 본 연구에 참여하였다. 나이와 체질량지수, 혈압, 혈청 지질농도를 보정한 후 당뇨 환자군에서 PBMC Lp-PLA_2 활성도와 C 반응단백질(hs-CRP), 종양괴사인자(TNF-α)가 대조군보다 더 높았다. 대조군과 비교하여 당뇨 환자군에서 11개의 PBMC 대사체 (6개의 아미노산, L-pyroglutamic acid, palmitic amide, oleamide, 2개의 lysoPCs) 이 더 높았다. 당뇨 환자군에게서 PBMC Lp-PLA_2 은 PBMC lysoPCs 및 염증지표(TNF-α, hs-CRP, Lp-PLA_2 활성도)와 유의적인 양의 상관관계를 보였다. 당뇨 환자군와 대조군의 혈장 대사체를 비교해보면 2개의 아미노산이 유의적으로 증가하였고, 5개의 lysoPCs만이 유의적으로 감소하였다. 본 연구를 통하여 당뇨환자와 정상인의 PBMC 대사체가 유의적으로 다르다는 것을 보였다. 이는 혈장 대사체보다 더 큰 차이이다. 이 결과로 PBMC가 당뇨의 염증 병태생리를 이해하는데 유용한 지표가 될 수 있을 것으로 사료된다.

핵심되는말: 제2형 당뇨, 당뇨전단계, 말초혈액단핵구 대사체, 혈장 대사체
염증지표, Lp-PLA_2, TNF-α, lysoPCs, hs-CRP